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(54) Title: USE OF CYTOKINES AND MITOGENS TO INHIBIT GRAFT VERSUS HOST DISEASE		
(57) Abstract <p>Disclosed is a method for inducing T cell tolerance in a sample of <i>ex vivo</i> peripheral blood mononuclear cells (PBMCs), comprising the addition to said cells of immunosuppressive agents such as IL-10 and TGF-beta. Also described is a kit for carrying out such a method and the relevance of the latter for instance for suppressing Graft Versus Host Disease (GVHD) in patients that have received allogeneic bone marrow transplants.</p>		

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USE OF CYTOKINES AND MITOGENS TO INHIBIT GRAFT VERSUS HOST DISEASE

FIELD OF THE INVENTION

5 The field of the invention is generally related to pharmaceutical agents useful in treating graft-versus-host disease (GVHD) in patients that have received allogenic bone marrow transplants.

BACKGROUND OF THE INVENTION

10 Organ transplantation is now used with great success to improve the quality of human life. Substantial progress has been made in using kidneys, hearts, and livers from unrelated individuals. However, transplantation of hematopoietic stem cells from an unrelated (or allogeneic) donor is a more complicated endeavor. Here multipotent stem cells which have the capacity to regenerate all the blood-forming elements and the immune system are harvested from bone marrow or peripheral blood from one individual are transferred to another. However, histocompatibility differences between donor and recipient results in a higher incidence of transplant-related complications, and has limited the use of this procedure (Forman et al., *Blackwell Scientific Publications*, 1994).

15

It is unfortunate that only few individuals are candidates for allogeneic hematopoietic stem cell transplantation at the present time because the spectrum of diseases treatable by this procedure has steadily increased. These diseases now include hematologic malignancies such as the acute or

chronic leukemias, multiple myeloma, myelodysplastic syndromes; lymphomas; and the severe anemias such as aplastic anemia or thalassemia.

5 Allogeneic stem cell transplantation begins with treatment of the recipient with a highly immunosuppressive conditioning regimen. This is most commonly accomplished with high doses of chemotherapy and radiation which effectively kill all the blood forming elements of the bone marrow. Besides preparing the recipient bone marrow for donor stem cell transplantation, the conditioning regimen serves to kill much of the malignancy that remains in the body. The period of time between the completion of the conditioning regimen, and engraftment of the donor stem cells is the most
10 dangerous for the recipient. It is during this time that the patient is completely immunocompromised and susceptible to a host of life-threatening infections. This vulnerability persists until the grafted donor stem cells proliferate and differentiate into the needed white blood cells and immune cells needed to combat infections.

15 Moreover, donor stem cell preparations generally contain immune cells called T lymphocytes. Unless the donor stem cells originate from an identical twin the transferred T cells turn against the recipient's tissues and trigger a deadly ailment called graft versus host disease (or GVHD). This is because the donor T lymphocytes recognize histocompatibility antigens of the recipient as foreign and respond by causing multi-organ dysfunction and destruction.

20 Current techniques of immunosuppression have made allogeneic stem cell transplantation from a related, histocompatible (HLA-matched) donor much safer than it once was. Allogeneic stem cell transplantation from an unrelated, HLA-matched donor is commonly complicated by serious, often fatal GVHD. The threat of GVHD is even higher when the stem cell donor is HLA mismatched.

25 Since only 30% of patients in need of allogeneic stem cells will have a sibling with identical histocompatibility antigens (Dupont, B., *Immunol Reviews* 157:12, 1997), there is a great need to make HLA-matched unrelated, and HLA-mismatched transplantation a safer procedure. There have

been two principal approaches to resolving this problem. The first has been to deplete the graft of contaminating T lymphocytes and the second has been to inactivate the T cells so they cannot attack the recipient.

5 In the 1970's it became evident that ex-vivo removal of mature T lymphocytes from a bone marrow graft prior to transplantation dramatically decreased or prevented GVHD in animals receiving marrow grafts across major histocompatibility barriers (Radt, H. J. *Immunol* 4:25-29, 1974; and 4 Vallera et al., *Transplantation* 31:218-222, 1981). However, with T cell depletion the incidence of graft failure, graft rejection, relapse of leukemia, and viral-induced lympho-proliferative disease markedly increased (Martin et al. *Blood* 66:664-672, 1985; 6 Patterson et al. *Br J Hematol* 63:221-230, 1986; Goldman et al. *Ann Intern Med* 108:806-814, 1988; and Lucas et al. *Blood* 87:2594-2603, 1996). Thus, the
10 transplantation of donor T cells on the stem cells has beneficial as well as deleterious effects. One needs the facilitating effect of the T cells on the engraftment of stem cells and the now well recognized graft-versus-tumor effects, but not graft-versus host disease.

Several approaches have been used to decrease T cell activation. These include: 1) *in vivo*
15 immunosuppressive effects of drugs such as FK506 and rapamycin (Blazar et al. *J. Immunol* 153:1836-1846, 1994; Dupont et al. *J. Immunol* 144:251-258, 1990; Morris, *Ann NY Acad Sci* 685:68-72, 1993; and Blazar et al. *J Immunol* 151:5726-5741, 1993); 2) the *in vivo* targeting of GVHD-reactive T cells using intact and F(ab')₂ fragments of monoclonal antibodies(mAb) reactive against T cell determinants or mAb linked to toxins (Gratama et al. *Am J Kidney Dis* 11:149-152, 1984; Hiruma et al. *Blood* 79:3050-3058, 1992; Anasetti et al. *Transplantation* 54:844-851, 1992; Martin et al.
20 *Bone Marrow Transplant* 3:437-444, 1989); 3) inhibition of T cell signaling via either IL-2/cytokine receptor interactions (Herve et al. *Blood* 76:2639-2640, 1990) or the inhibition of T cell activation through blockade of co-stimulatory or adhesogenic signals (Boussiotis et al. *J Exp Med* 178:1753-1763, 1993; Gribben et al. *Blood* 97:4887-4893, 1996; and Blazar et al. *Immunol Rev* 157:79-90, 1997); 4) the shifting of the balance between acute GVHD-inducing T helper-type 1 T cells to
25 anti-inflammatory T helper-type 2 T cells via the cytokine milieu in which these cells are generated

(Krenger et al. *Transplantation* 58:1251-1257, 1994; Blazar et al. *Blood* 88:247, 1996, abstract; Krenger et al. *J Immunol* 153:585-593, 1995; Fowler et al. *Blood* 84:3540-3549, 1994); 5) the regulation of alloreactive T cell activation by treatment with peptide analogs which affect either T cell receptor/major histocompatibility complex (MHC) interactions, class II MHC/CD4 interactions, or class I MHC/CD8 interactions (Townsend and Korngold (unpublished data)); and 6) the use of gene therapy to halt the attack of donated cells on the recipient's tissues (Bonini et al. *Science* 276:1719-24, 1997).

There is suggestive evidence that the T lymphocytes from non-identical donors can become tolerant to the recipient's tissues. Unlike patients who receive solid organ allografts for whom life-long immunosuppressive therapy is needed to control chronic rejection, there is evidence of immunologic tolerance with stem cell allografts. The majority of these patients can be withdrawn from immune suppression without further evidence of GVHD (Storb et al. *Blood* 80:560-561, 1992; and Sullivan et al. *Semin Hematol* 28:250-259, 1992).

Immunologic tolerance is a specific state of non-responsiveness to an antigen. Immunologic tolerance generally involves more than the absence of an immune response; this state is an adaptive response of the immune system, one meeting the criteria of antigen specificity and memory that are the hallmarks of any immune response. Tolerance develops more easily in fetal and neonatal animals than in adults, suggesting that immature T and B cells are more susceptible to the induction of tolerance. Moreover, studies have suggested that T cells and B cells differ in their susceptibility to tolerance induction. Induction of tolerance, generally, can be by clonal deletion or clonal anergy. In clonal deletion, immature lymphocytes are eliminated during maturation. In clonal anergy, mature lymphocytes present in the peripheral lymphoid organs become functionally inactivated.

Following antigenic challenge stimulation, T cells generally are stimulated to either promote antibody production or cell-mediated immunity. However, they can also be stimulated to inhibit these immune responses instead. T cells with these down-regulatory properties are called "suppressor cells".

Although it is known that T suppressor cells produce cytokines such as transforming growth factor beta (TGF-beta), interleukin 4 (IL-4) or interleukin (IL-10) with immunosuppressive effects, until recently the mechanisms responsible for the generation of these regulatory cells have been poorly understood. It was generally believed that CD4+ T cells induce CD8- T cells to develop

5 down-regulatory activity and that interleukin 2 (IL-2) produced by CD4+ cells mediates this effect.

Although most immunologists agree that IL-2 has an important role in the development of T suppressor cells, whether this cytokine works directly or indirectly is controversial (Via et al.

International Immunol 5:565-572, 1993; Fast, *J Immunol* 149:1510-1515, 1992; Hirohata et al. *J*

Immunol 142:3104-3112, 1989; Taylor, *Advances Exp Med Biol* 319:125-135, 1992; and Kinter et al.,

10 *Proc. Natl. Acad. Sci. USA* 92:10985-10989, 1995). Recently, IL-2 has been shown to induce CD8+ cells to suppress HIV replication in CD4-T cells by a non-lytic mechanism. This effect is cytokine mediated, but the specific cytokine with this effect has not been identified (Barker et al. *J Immunol* 156:4476-83, 1996; and Kinter et al. *Proc Natl Acad Sci USA* 99:10985-9 1995).

A model using human peripheral blood lymphocytes to study T cell/B cell interactions in the absence of
15 other accessory cells has been developed (Hirokawa et al. *J. Immunol.* 149:1859-1866, 1977). With this model it was found that CD4+ T cells by themselves generally lacked the capacity to induce CD8+ T cells to become potent suppressor cells. The combination of CD8+ T cells and NK cells, however, induced strong suppressive activity (Gray et al. *J Exp Med* 180:1937-1942, 1994). It was then demonstrated that the contribution of NK cells was to produce TGF-beta in its active form. It was then
20 reported that a small non-immunosuppressive concentration (10-100 pg/ml) of this cytokine served as a co-factor for the generation of strong suppressive effects on IgG and IgM production (Gray et al. *J Exp Med* 180:1937-1942, 1994). Further, it was demonstrated that NK cells are the principal lymphocyte source of TGF-beta (Gray et al. *J Immunol*, 160:2248-2254, 1998).

TGF-beta is a multifunctional family of cytokines important in tissue repair, inflammation and
25 immunoregulation (Border et al. *J Clin Invest* 90:1-7, 1992; and Sporn et al. *J Cell Biol* 105:1039-1045, 1987). TGF-beta is unlike most other cytokines in that the protein released is biologically inactive and

unable to bind to specific receptors (Massague, *Cell* 69:1067-1070, 1992). The conversion of latent to active TGF-beta is the critical step which determines the biological effects of this cytokine.

There is some evidence that NK cell-derived TGF-beta has a role in the prevention of GVHD.

5 Whereas the transfer of stem cells from one strain of mice to another histocompatibility mismatched strain resulted in death of all recipients from GVHD within 19 days, the simultaneous transfer of NK cells from the donor animals completely prevented this consequence. All the recipient mice survived indefinitely. This therapeutic effect, however, was completely blocked by antagonizing the effects of TGF-beta by the administration of a neutralizing antibody (Murphy et al. *Immunol Rev* 157:167-176, 1997).

10 It is very likely, therefore, that the mechanism whereby NK cell-derived TGF-beta prevented GVHD is similar to that described by Horwitz et al. in the down-regulation of antibody production. In each case NK cell-derived TGF-beta was responsible for the generation of suppressor lymphocytes that blocked these respective immune responses. The mouse study is of particular interest since the histocompatibility differences between genetically disparate inbred mice strains would mirror that of
15 unrelated human donors. A modification of this strategy, therefore might overcome GVHD in mismatched humans.

Anti-CD2 monoclonal antibodies and other constructs that bind to the CD2 co-receptor have been shown to be immunosuppressive. It has now been demonstrated that at least one mechanism to explain this immunosuppressive effect is by inducing the production of TGF-beta (Gray et al. *J Immunol*, 160:2248-2254, 1998).
20

One strategy to prevent GVHD would be to isolate and transfer NK cells along with the stem cells. Another would be to treat the immunocompromised recipient who has received allogeneic stem cells with TGF-beta, anti-CD2 monoclonal antibodies, IL-2 or a combination of these cytokines. The first strategy would be difficult because NK cells comprise only 10 to 20% of total lymphocytes so that it

would be difficult to harvest a sufficient number of cells for transfer. The second strategy is limited by the systemic toxic side effects of these monoclonal antibodies and cytokines. IL-2 and TGF-beta have numerous effects on different body tissues and are not very safe to deliver to a patient systemically. What is needed, therefore, is a way to induce mammalian cells to suppress the development of GVHD

5 *ex vivo*.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides methods for inducing T cell tolerance in a sample of *ex vivo* peripheral blood mononuclear cells (PBMCs) comprising adding a suppressive composition to the cells. The suppressive composition can be IL-10, TGF- β , or a mixture.

10 In an additional aspect, the present invention provides methods for treating donor cells to ameliorate graft versus host disease in a recipient patient. The methods comprise removing peripheral blood mononuclear cells (PBMC) from a donor, and treating the cells with a suppressive composition for a time sufficient to induce T cell tolerance. The cells are then introducing to a recipient patient. The PBMCs can be enriched for CD8+ cells, if desired. The methods may additionally comprising adding

15 the treated cells to donor stem cells prior to introduction into the patient.

In an additional aspect, the invention provides kits for the treatment of donor cells comprising a cell treatment container adapted to receive cells from a donor and at least one dose of a suppressive composition. The kits may additionally comprising written instructions and reagents. The cell treatment container may comprise a sampling port to enable the removal of a fraction of the cells for

20 analysis, and an exit port adapted to enable transport at least a portion of the cells to a recipient patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict that TGF- β can upregulate expression of CD40 Ligand (CD40L) on T cells. Purified T cells were stimulated with PMA (20 ng/ml) and ionomycin (5 μ M) in the presence or absence of TGF- β . After 6 hours the cells were stained with anti-CD40L antibodies. In the absence of TGF- β , there were 30% positive cells (solid line, panel A). With 100 pg/ml of TGF- β , 66% of the cells were positive (solid line, panel B). The dotted line in both panels is the reactivity of a control antibody.

Figures 2A, 2B, 2C and 2D depict that TGF- β increases TNF- α expression by CD8+ cells. Purified CD8+ cells were stimulated for 24 hours with Con A (5 μ g/ml) \pm TGF- β (10 pg/ml) \pm IL-2 (10 U). During the last 6 hours, monensin (2 μ M) was also present to prevent cytokine release. The cells were first stained with anti-CD69 to distinguish the activated cells. Then the cells were fixed (4% paraformaldehyde), permeabilized (0.1% saponin) and stained with anti-TNF- α antibodies.

Figures 3A and 3B depict TGF- β enhances IL-2 expression by T cells. Purified T cells were stimulated in the presence or absence of TGF- β (1 ng/ml). In the absence of TGF- β , 36% of the cells were positive (panel A, solid line) whereas with TGF- β , 53% were positive (panel B, solid line).

Figures 4A, 4B and 4C depict that TGF- β can enhance or inhibit cytotoxic activity. In panels A and B, purified T cells were cultured with irradiated allogenic stimulator cells in the presence or absence of the indicated cytokines. After 48 hours, the cells were washed and after a further 3 days, assayed for cytotoxic activity against 51 Cr-labelled stimulator ConA blasts. In panel C, purified CD8+ cells were cultured with irradiated allogenic cells in the presence or absence of TGF- β (10 pg/ml) or IL-12 (100 U). After 48 hours, the cells were washed and added to autologous T cells and irradiated allogenic cells. After 5 days of culture, cytotoxic activity was determined using 51 Cr-labelled stimulator ConA blasts as target cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows for the transfer of histoincompatible stem cells to humans with a variety

of malignant or hereditary diseases using a method to prevent life-threatening graft-versus-host disease. This is accomplished by treatment of donor cells with a combination of mitogens and cytokines ex-vivo. The particular advantage of this procedure is that it avoids the removal of donor T cells which facilitate stem cell engraftment and that have the potential to attack any remaining malignant cells. Once a state of tolerance between donor and host has been achieved, non-conditioned donor T cells can be transferred to maximize the beneficial graft-versus-tumor immune response.

This strategy is unlike almost all other treatment modalities currently in use. These cytokines and mitogens described would have severe toxic side effects if administered *in vivo*. The ex-vivo protocol described avoids these side effects. The ability to successfully engraft histoincompatible stem cells for treatment of life-threatening diseases would be a milestone in medicine.

In addition, a further advantage of the present invention is that it may avoid or minimize the very toxic immunosuppressive medicines that must be given to the recipient to prevent GVHD. These medicines also block the ability of the donor-derived lymphocytes which repopulate the immune system of the recipient from becoming "educated" to their new host. Therefore, it is difficult to stop the immunosuppressive drugs, unless an alternative treatment such as the present invention is used.

The strategy of the present invention is to suppress GVHD by both suppressing T cell activation and inducing a tolerant state in the donor cells, which prevents the donor cells from attacking recipient cells. Surprisingly, the methods outlined herein result in not only the suppression of the treated cells but additionally induces them to prevent other donor cells from killing recipient cells as well, i.e. they become tolerant. That is, the methods outlined herein not only decrease the capacity of the donor's cells to attack the recipient's cells, but induces some of the donor's cells to assume a surveillance role and prevent other donor cells from mounting an immune attack against the recipient host. The net result is for the donor lymphocytes to be tolerant to the histocompatibility antigens of the recipient, but does not impair the ability of the the new lymphocytes to attack tumor cells.

Another significant potential advantage of this strategy is a low probability of serious adverse side effects. Since only trace amounts of suppressive compositions such as cytokines will be returned to the patient, there should be minimal toxicity.

5 Accordingly, the present invention is drawn to methods of treating donor cells for transplantation into a recipient that comprise removing peripheral blood mononuclear cells (PBMCs) from the donor and treating the cells with a composition that is on one hand suppressive, but on the other hand generates surveillance cells to prevent an immune attack.

10 The present invention shows that the treatment of the donor cells by a suppressive composition blocks an immune attack against the recipient's cells. Without being bound by theory, it appears that there are several ways the methods of the invention may work. First of all, the donor cells are activated to become tolerant to the recipient's cells. Secondly, the donor CD8+ cells get activated to become regulatory cells, to prevent other donor cells from killing recipient cells. These results lead to amelioration of a GVH response. Without being bound by theory, it appears that the inhibition of cytotoxic activity may occur as a result of the effects TGF- β on the cells; as depicted in the figures, the
15 addition of TGF- β causes the upregulation of CD40L on T cells, increases TNF- α expression by CD8+ cells, and enhances IL-2 expression.

Thus, in a preferred embodiment, the present invention induces tolerance in the donor cells to recipient tissue, thus avoiding GVHD, by treating them with a suppressive composition *ex vivo*.

20 Accordingly, the present invention provides methods of treating donor cells to induce or establish tolerance to recipient cells prior to transplantation into a recipient patient to decrease or eliminate a graft-versus-host response. By "T cell tolerance" herein is meant immune non-responsiveness to the recipient, i.e. a tolerance to the histocompatibility antigens of the recipient. Without being bound by theory, this may be due to anergy or death of the T cells. Preferably, the T cells retain the ability to recognize other antigens as foreign, to facilitate tumor killing and general immunological responses to

foreign antigens.

Using the methods outlined herein, a GVH response is suppressed or treated. By "treating" GVHD herein is meant that at least one symptom of the GVHD is ameliorated by the methods outlined herein. This may be evaluated in a number of ways, including both objective and subjective factors on the part
5 of the patient as is known in the art. For example, GVHD generally exhibits a skin rash, an abnormality in liver function studies, fever, general symptoms including fatigue, anemia, etc.

By "patient" herein is meant a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters;
10 and primates.

The methods provide for the removal of blood cells from a patient. In general, peripheral blood mononuclear cells (PBMCs) are taken from a patient using standard techniques. By "peripheral blood mononuclear cells" or "PBMCs" herein is meant lymphocytes (including T-cells, B-cells, NK cells, etc.) and monocytes. As outlined more fully below, it appears that the main effect of the suppressive
15 composition is to enable CD8+ T cells to become tolerant. Accordingly, the PBMC population should comprise CD8+ T cells. Preferably, only PBMCs are taken, either leaving or returning red blood cells and polymorphonuclear leudocytes to the patient. This is done as is known in the art, for example using leukaphoresis techniques. In general, a 5 to 7 liter leukaphoresis step is done, which essentially removes PBMCs from a patient, returning the remaining blood components. Collection of the cell
20 sample is preferably done in the presence of an anticoagulant such as heparin, as is known in the art.

In general, the sample comprising the PBMCs can be pretreated in a wide variety of ways. Generally, once collected, the cells can be additionally concentrated, if this was not done simultaneously with collection or to further purify and/or concentrate the cells. The cells may be washed, counted, and resuspended in buffer.

The PBMCs are generally concentrated for treatment, using standard techniques in the art. In a preferred embodiment, the leukophoresis collection step results a concentrated sample of PBMCs, in a sterile leukopak, that may contain reagents or doses of the suppressive composition, as is more fully outlined below. Generally, an additional concentration/purification step is done, such as Ficoll-Hypaque density gradient centrifugation as is known in the art.

In a preferred embodiment, the PBMCs are then washed to remove serum proteins and soluble blood components, such as autoantibodies, inhibitors, etc., using techniques well known in the art. Generally, this involves addition of physiological media or buffer, followed by centrifugation. This may be repeated as necessary. They can be resuspended in physiological media, preferably AIM-V serum free medium (Life Technologies) (since serum contains significant amounts of inhibitors of TGF- β) although buffers such as Hanks balanced salt solution (HBBS) or physiological buffered saline (PBS) can also be used.

Generally, the cells are then counted; in general from 1×10^8 to 2×10^9 white blood cells are collected from a 5-7 liter leukophoresis step. These cells are brought up roughly 200 mls of buffer or media.

In a preferred embodiment, the PBMCs may be enriched for one or more cell types. For example, the PBMCs may be enriched for CD8+ T cells, CD4+ T cells or, in the case of stem cell isolation as is more fully described below, CD34+ stem cells. This is done as is known in the art, as described in Gray *et al.* (1998), *J. Immunol.* 160:2248, hereby incorporated by reference. Generally, this is done using commercially available immunoabsorbent columns, or using research procedures (the PBMCs are added to a nylon wool column and the eluted, nonadherent cells are treated with antibodies to CD4, CD16, CD11b, and CD74, followed by treatment with immunomagnetic beads, leaving a population enriched for CD8+ T cells). In one embodiment, cell populations are enriched for CD8+ cells, as these appear to be the cells most useful in the methods of the invention. However, one advantage of using PBMCs is that other cell types within the PBMC population produce IL-10, thus decreasing or even eliminating the requirement of the suppressive composition comprising IL-10.

Once the cells have undergone any necessary pretreatment, the cells are treated with a suppressive composition. By "treated" in this context herein is meant that the cells are incubated with the suppressive composition for a time period sufficient to result in T cell tolerance, particularly when transplanted into the recipient patient. The incubation will generally be under physiological temperature.

By "suppressive composition" or "tolerance composition" is meant a composition that can induce T cell tolerance. Generally, these compositions are cytokines. Suitable suppressive compositions include, but are not limited to, IL-10, IL-2 and TGF- β . A preferred suppressive composition is a mixture of IL-10 and TGF- β .

The concentration of the suppressive composition will vary on the identity of the composition, but will generally be at physiologic concentration, i.e. the concentration required to give the desired effect, i.e. an enhancement of specific types of regulatory cells. In a preferred embodiment, TGF- β is used in the suppressive composition. By "transforming growth factor - β " or "TGF- β " herein is meant any one of the family of the TGF- β s, including the three isoforms TGF- β 1, TGF- β 2, and TGF- β 3; see Massague, (1980), *J. Ann. Rev. Cell Biol* 6:597. Lymphocytes and monocytes produce the β 1 isoform of this cytokine (Kehrl *et al.* (1991), *Int J Cell Cloning* 9:438-450). The TGF- β can be any form of TGF- β that is active on the mammalian cells being treated. In humans, recombinant TGF- β is currently preferred. A human TGF- β 2 can be purchased from Genzyme Pharmaceuticals, Farmington, MA. In general, the concentration of TGF- β used ranges from about 2 picograms/ml of cell suspension to about 2 nanograms, with from about 10 pg to about 500 pg being preferred, and from about 50 pg to about 150 pg being especially preferred, and 100 pg being ideal.

In a preferred embodiment, IL-10 is used in the suppressive composition. The IL-10 can be any form of IL-10 that is active on the mammalian cells being treated. In humans, recombinant IL-10 is currently preferred. Recombinant human IL-10 can be purchased. In general, the concentration of IL-10 used ranges from about 1 U/ml of cell suspension to about 100, with from about 5 to about 50 being

preferred, and with 10 U/ml being especially preferred.

In a preferred embodiment, IL-2 is used as the suppressive composition. The IL-2 can be any form of IL-2 that is active on the mammalian cells being treated. In humans, recombinant IL-2 is currently preferred. Recombinant human IL-2 can be purchased from Cetus, Emeryville, CA. In general, the concentration of IL-2 used ranges from about 1 Unit/ml of cell suspension to about 100 U/ml, with from about 5 U/ml to about 25 U/ml being preferred, and with 10 U/ml being especially preferred. In a preferred embodiment, IL-2 is not used alone.

In a preferred embodiment, TGF- β can be used alone as the suppressive composition. Alternate preferred embodiments utilize IL-10 alone, combinations of TGF- β , IL-10 and IL-2, with the most preferred embodiment utilizing a mixture of TGF- β and IL-10.

The suppressive composition is incubated with the donor cells and a population of irradiated PMBC recipient cells (harvested as outlined above). The recipient cells are irradiated so that they cannot attack the donor cells, but will stimulate the donor cells to become tolerant to the recipient cells. The incubation occurs for a period of time sufficient to cause an effect, generally from 4 hours to 96 hours, although both shorter and longer time periods are possible.

In one embodiment, treatment of the donor cells with the suppressive composition is followed by immediate transplantation into the recipient patient, generally after the cells have been washed to remove the suppressive composition.

In a preferred embodiment, a second step is done. In this embodiment, after the donor cells have been conditioned or treated with the suppressive composition, they may be frozen or otherwise stored. Then a second step comprising obtaining a population of donor hematopoietic stem cells from aspirated bone marrow or PBMCs. Stem cells comprise a very small percentage of the white blood cells in blood, and are isolated as is known in the art, for example as described in U.S. Patent Nos.

5,635,387 and 4,865,204, both of which are incorporated by reference in their entirety, or harvested using commercial systems such as those sold by Nexell. As outlined above, CD34+ stem cells can be concentrated using affinity columns; the eluted cells are a mixture of CD34+ stem cells and lymphocytes. The contaminating lymphocytes are generally be removed using known techniques such as staining with monoclonal antibodies and removal using conventional negative selection procedures.

Once the CD34+ stem cells have been isolated, they may be mixed with the donor cells previously treated with the suppressive composition and immediately introduced into the recipient patient.

In one embodiment, the cells are treated for a period of time, washed to remove the suppressive composition, and may additionally be reincubated. The cells are preferably washed as outlined herein to remove the suppressive composition. Further incubations for testing or evaluation may also be done, ranging in time from a few hours to several days. If evaluation of any cellular characteristics prior to introduction to a patient is desirable, the cells may be incubated for several days to several weeks to expand numbers of suppressor cells.

Once the cells have been treated, they may be evaluated or tested prior to transplantation into the patient. For example, a sample may be removed to do: sterility testing; gram staining, microbiological studies; LAL studies; mycoplasma studies; flow cytometry to identify cell types; functional studies, etc. Similarly, these and other lymphocyte studies may be done both before and after treatment. A preferred analysis is a test using labeled recipient cells; incubating the treated tolerant donor cells with a labeled population of the recipient cells will verify that the donor cells are tolerant and won't kill the recipient cells.

In a preferred embodiment, the treatment results in the conditioning of the T cells to become non-responsive to histocompatibility antigens of the recipient so that GVHD is prevented.

In a preferred embodiment, prior to transplantation, the amount of total or active TGF- β can also be tested. As noted herein, TGF- β is made as a latent precursor that is activated post-translationally.

After the cell treatment, the donor cells are transplanted into the recipient patient. The MHC class I and class II profiles of both the donor and the recipient are determined. Preferably, a non-related
5 donor is found that preferably matches the recipients HLA antigens, but may mismatch at one or more loci if a matched donor cannot be identified. The recipient patient has generally undergone bone marrow ablation, such as a high dose chemotherapy treatment, with or without total body irradiation.

The donor cells are transplanted into the recipient patient. This is generally done as is known in the art, and usually comprises injecting or introducing the treated cells into the patient as will be
10 appreciated by those in the art. This may be done via intravascular administration, including intravenous or intraarterial administration, intraperitoneal administration, etc. For example, the cells may be placed in a 50 ml Fenwall infusion bag by injection using sterile syringes or other sterile transfer mechanisms. The cells can then be immediately infused via IV administration over a period of time, such as 15 minutes, into a free flow IV line into the patient. In some embodiments, additional
15 reagents such as buffers or salts may be added as well.

After reintroducing the cells into the patient, the effect of the treatment may be evaluated, if desired, as is generally outlined above and known in the art.

The treatment may be repeated as needed or required. After a period of time, the leukemic cells may reappear. Because the donor lymphocytes are now tolerant to the recipient's cells, the patient now
20 receives a transfusion of unconditioned donor lymphocytes which recognize the leukemic cells as foreign and kill these cells.

In a preferred embodiment, the invention further provides kits for the practice of the methods of the invention, *i.e.*, the incubation of the cells with the suppressive compositions. The kit may have a

number of components. The kit comprises a cell treatment container that is adapted to receive cells from a donor. The container should be sterile. In some embodiments, the cell treatment container is used for collection of the cells, for example it is adaptable to be hooked up to a leukapheresis machine using an inlet port. In other embodiments, a separate cell collection container may be used.

- 5 The form and composition of the cell treatment container may vary, as will be appreciated by those in the art. Generally, the container may be in a number of different forms, including a flexible bag, similar to an IV bag, or a rigid container similar to a cell culture vessel. It may be configured to allow stirring. Generally, the composition of the container will be any suitable, biologically inert material, such as glass or plastic, including polypropylene, polyethylene, etc. The cell treatment container may have
- 10 one or more inlet or outlet ports, for the introduction or removal of cells, reagents, suppressive compositions, etc. For example, the container may comprise a sampling port for the removal of a fraction of the cells for analysis prior to introduction into the recipient patient. Similarly, the container may comprise an exit port to allow introduction of the cells into the recipient patient; for example, the container may comprise an adapter for attachment to an IV setup.
- 15 The kit further comprises at least one dose of a suppressive composition. "Dose" in this context means an amount of the suppressive composition such as cytokines, that is sufficient to cause an effect. In some cases, multiple doses may be included. In one embodiment, the dose may be added to the cell treatment container using a port; alternatively, in a preferred embodiment, the dose is already present in the cell treatment container. In a preferred embodiment, the dose is in a lyophilized
- 20 form for stability, that can be reconstituted using the cell media, or other reagents.

In some embodiments, the kit may additionally comprise at least one reagent, including buffers, salts, media, proteins, drugs, etc. For example, mitogens can be included.

In some embodiments, the kit may additionally comprise written instructions for using the kits.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are
5 incorporated by reference in their entirety.

EXAMPLES

Example 1

Donor lymphocyte *ex vivo* treatment to prevent an immune attack against
blood cells from an unrelated recipient

10 A blood sample from a donor was obtained and lymphocytes prepared by density gradient centrifugation. T cells were prepared using a conventional negative selection procedure. These T cells were conditioned to prevent them from attacking the recipient cells. For this conditioning, the CD8+ T cells were mixed with irradiated stimulator cells from the recipient. The stimulator cells were derived from T cell-depleted blood cells from the recipient. The mixture of donor T cells and recipient
15 stimulator cells were cultured for 48 hours with different concentrations of one or more cytokines. In this example the cytokines were TGF- β and IL-10. This procedure abolished the potential of the donor T cells to kill recipient cells, in Figure 4B.

To test the ability of the donor T cells to recognize and kill recipient blood cells, the donor T cells were cultured with irradiated stimulator cells for 5 days. Then the donor cells were cultured for 4 hours with
20 a sample of recipient radiolabeled blood cells. When the recipient's cells are killed they release radioisotope into the culture medium. By determining the amount of radioisotope released, one can calculate the percentage of cells killed. In the standard cytotoxicity assay shown in Figure 1, donor cells were cultured with labeled recipient cells in 30 to 1, 15 to 1, and 7.5 to 1 ratios. These combinations of donor and recipient cells are called effector to target ratios. Killing is indicated by the

various symbols. As expected, maximum killing was seen at the highest effector to target cell ratio. In panel A, the open circles shows that if 30 donor cells were mixed with 1 recipient cell, 40 percent of the recipient cells were killed. When donor T cells were conditioned with very small concentrations of TGF- β (0.01 or 0.1 nanograms per ml), they had no effect on killing. However, if the T cells were treated with 1 nanogram per ml of TGF- β , the killing of recipient cells decreased by 50 percent. Panel B shows that if the T cells were treated with IL-10, killing also decreased by 50%. If the T cells had been conditioned with both IL-10 and TGF- β at 1 nanogram per ml, these cells completely blocked the killing of recipient cells; killing was almost undetectable. Various combinations of mitogens, cytokines, and monoclonal antibodies can be used to make T cells non-responsive.

Example 2

CD8⁺ T cells from the donor conditioned *ex vivo* to prevent other donor's T cells from mounting an attack against blood cells from an unrelated recipient.

A blood sample from a donor was obtained and lymphocytes prepared. CD8⁺ T cells were mixed with irradiated stimulator cells of the recipient and either TGF- β (picograms per ml) or IL-12 100 U/ml. IL-12 is known to enhance the ability of CD8⁺ T cells to develop the capacity to kill. Here IL-12 was used to show that a given population of CD8⁺ cells can be induced to kill or to block killing depending upon how they are activated. Other CD8⁺ cells were cultured in culture medium only as a control (CD8 med).

The CD8⁺ T cells, the stimulator cells and the cytokines were cultured for 48 hours and the cytokines were removed from the cultures by washing. This procedure not only abolished the potential of the TGF- β conditioned CD8⁺ cells to kill the recipient cells, but also induced them to prevent other donor T cells from killing the recipient cells (Figure 4C).

To enable the donor T cells to recognize and kill recipient blood cells, the donor cells were cultured

with irradiated stimulator cells for five days. Then the donor cells were cultured for 4 hours with a sample of recipient radiolabeled blood cells. The open circles show the level of donor cells of recipient cells when no CD8+ cells were added. At a 30:1 effector to target cell ration, 30% of the recipient's cells were killed. If CD8+ cells that had been cultured for 48 hours without cytokines were added, there was no change in the killing (CD8+ +Med, solid circles). If the CD8+ cells had been conditioned with TGF- β , killing was *suppressed* by about 50%. However, the CD8+ T cells conditioned with TGF- β not only did not kill, but the decreased levels of cytotoxicity indicate that they blocked the ability of other T cells to kill blood cells of the recipient.

Example 3

Treating a patient with chronic myelocytic leukemia with the stem cells
from a histoincompatible donor: tolerization with mitogens

The harvested PBMC of the donor are placed in a sterile container in HBBS as in Example 1. The cells are then incubated with mitogens to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case the cells are incubated with physiological concentrations of concanavalin A (Con A) for 4 to 72 hours using standard incubation techniques. The concentration of Con A used can range from about 0.01 to about 10 micrograms/ml with 1 microgram/ml being presently preferred. Alternatively, SEB may be used as the mitogen at concentrations of 0.001 to 100 ng/ml.

The incubation of the mononuclear cells in the mitogen solution increases the population of T suppressor cells. These cells, when transferred to the recipient, will enable the stem cells to engraft without causing GVHD. Although it is not known how the mitogens work, it is believed to induce the production of TGF-beta by certain mononuclear cells in preparation, and the TGF-beta acts on T cells to become suppressor cells.

After the cells have been incubated with the mitogens, the cells are washed with HBBS to remove any mitogens that are in the solution. The cells are suspended in 200-500 ml of HBBS, mixed with the stem cells and administered to a patient with CML who has been treated with myeloablative agents to prepare the stem cells for engraftment.

- 5 Once the donor hematopoietic cells lymphocytes engraft in the recipient, and the patient again becomes healthy and free of leukemic cells. If the leukemic cells recur, the patient receives a transfusion of donor lymphocytes and the leukemic cells again disappear.

Example 4

Treating a patient with chronic myelocytic leukemia

- 10 with the stem cells from a histoincompatible donor: tolerization
 with anti-CD2 monoclonal antibodies.

- The harvested enriched stem cell preparation of the donor are placed in a sterile container in HBBS as in Example 1. The cells are then incubated with anti-CD2 monoclonal antibodies to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case, the
15 cells are incubated with anti-CD2 monoclonal antibodies for 4 to 72 hours using standard incubation techniques. The concentration of anti-CD2 monoclonal antibodies are 10 ng/ml to 10 ug/ml. Optionally, 1-1000 units of IL-2 can be added.

- The incubation of the mononuclear cells in the anti-CD2 solution increases the population of T suppressor cells. These cells, when transferred to the recipient will enable the stem cells to engraft
20 without causing GVHD. It is believed that incubation with anti-CD2 monoclonal antibodies induces the production of TGF-beta by certain mononuclear cells in preparation, and the TGF-beta acts on T cells to become suppressor cells.

After the cells have been incubated with the anti-CD2 monoclonal antibodies, the cells are washed

with HBBS to remove antibodies that are in the solution. The cells are suspended in 200-500 ml of HBBS mixed with the stem cells harvested previously and administered to a patient with CML who has been treated with myeloblastic agents to prepare the stem cells for engraftment.

Once the donor hematopoietic cells lymphocytes engraft in the recipient, and the patient again becomes healthy and free of leukemic cells. If the leukemic cells recur, the patient receives a transfusion of donor lymphocytes and the leukemic cells again disappear.

Example 5

Treating a patient with chronic myelocytic leukemia

With the stem cells from a histoincompatible donor: tolerization

With mitogens and cytokines.

The harvested PBMC of the donor are placed in a sterile container HBBS as in Example 1. The cells are then incubated with cytokines and mitogens to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case the cells are incubated with physiological concentrations of Con A or SEB, IL-2 or IL-10 and TGF-beta for 4 to 72 hours using standard incubation techniques.

After the cells have been incubated with the cytokines and mitogens, the cells are washed with HBBS to remove any cytokine and mitogen that are in the solution. The cells are suspended in 200-500 ml of HBBS mixed with the stem cells and administered to a patient with CML who has been treated with myeloabative agents to prepare the stem cells for engraftment.

Once the donor hematopoietic cells and lymphocytes engraft in recipient and the patient again becomes healthy and free of leukemic cells. If the leukemic cells recur, the patient receives a transfusion of donor lymphocytes and the leukemic cells again disappear.

Example 6

Treating a patient with chronic myelocytic leukemia

With the stem cells from a histoincompatible donor; tolerization

With a mitogen and cytokine.

5 The harvested PBMC of the donor are placed in a sterile container in HBBS as in Example 1. The cells are then incubated with a cytokine and a mitogen to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case the cells are incubated with physiological concentrations of ConA, and IL-2 for 4 to 72 hours using standard incubation techniques. In another case, SEB could be used.

10 After the cells have been incubated with the cytokines and mitogens, the cells are washed with HBBS to remove any cytokine and mitogen that are in the solution. The cells are suspended in 200-500 ml of HBBS mixed with stem cells and administered to a patient with CML who has been treated with myeloablative agents to prepare the stem cells for engraftment.

Example 7

15 **Treating a patient with chronic myelocytic leukemia**

Who has developed GVHD following the stem cell transplant.

In the instance that the initial procedure to prevent early or late GVHD following the stem cell transplant is not successful, this event will be managed BY transfer of a larger number of donor T cells that have been conditioned to become suppressor cells. Approximately 1×10^9 PBMCs obtained by leukopheresis are concentrated in a sterile leukopak; in Hanks balanced salt solution (HBBS). The PMMCs or separated CD8+ T cells (or the specific suppressor cell precursor subset CD8+CD45RA+C27+) prepared by immunoaffinity columns will be treated with antiCD2 monoclonal antibodies and/or mitogens and/or cytokines described above to condition them to become suppressor cells.

20

After incubation with the cytokines or mitogens for a period of time ranging from 4 hours to 72 hours, the cells are washed to remove the cytokines or mitogens and then are transferred to the recipient.

These conditioned T cells migrate to lymphoid organs and suppress the GVHD.

5 Besides chronic myelocytic leukemia, other hematologic malignancies such as acute and chronic leukemias, lymphomas, solid tumors such as breast carcinoma or renal cell carcinoma among a few , and non-malignant diseases such as severe anemias (thalassemia, sickle cell anemia) can be treated with mismatched allogeneic stem cells.

10 Another aspect of this invention is a kit to perform the cell incubation with the cytokines. The kit comprises a sterile incubating container with the appropriate concentration of cytokines preloaded within the container. In one embodiment of the kit, the cytokines are present in lyophilized form in the container. The container is preferably a bag, similar to an IV bag. The lyophilized cytokines are reconstituted with HBBS and then the cells are injected into the container and thoroughly mixed and incubated. In another embodiment of the invention the cytokines are already in solution within the container and all that has to be done is the injection of washed stem cell preparation and incubation.

CLAIMS

What is claimed is:

1. A method for inducing T cell tolerance in a sample of *ex vivo* peripheral blood mononuclear cells (PBMCs) comprising adding a suppressive composition to said cells.

5 2. A method for treating donor cells to ameliorate graft versus host disease in a recipient patient comprising:

a) removing peripheral blood mononuclear cells (PBMC) from a donor;

b) treating said cells with a suppressive composition for a time sufficient to induce T cell tolerance; and

10 c) introducing said cells to said patient.

3. A method according to claim 1 or 2 wherein said suppressive composition comprises TGF- β .

4. A method according to claim 1 or 2 wherein said suppressive composition comprises a mixture of IL-10 and TGF- β .

15 5. A method according to claim 2 wherein said method further comprises adding said cells to donor stem cells prior to introduction into said patient.

6. A kit for the treatment of donor cells comprising:

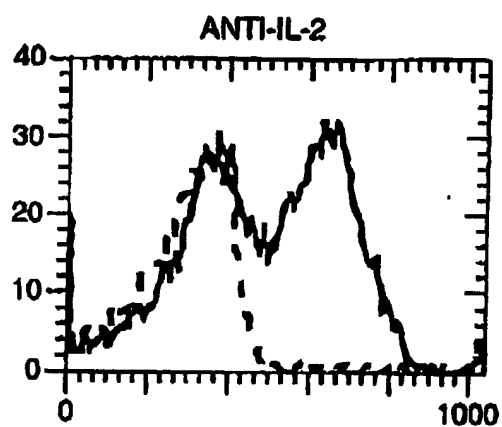
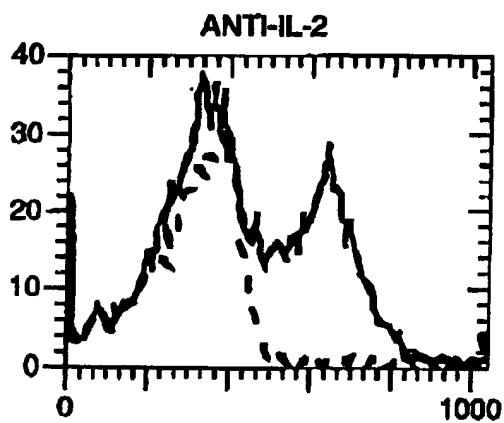
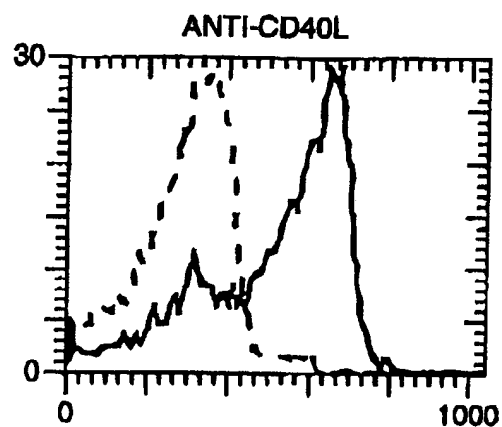
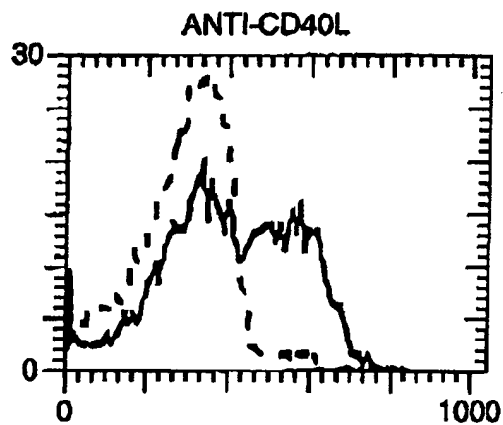
a) a cell treatment container adapted to receive cells from a donor; and

b) at least one dose of a suppressive composition.

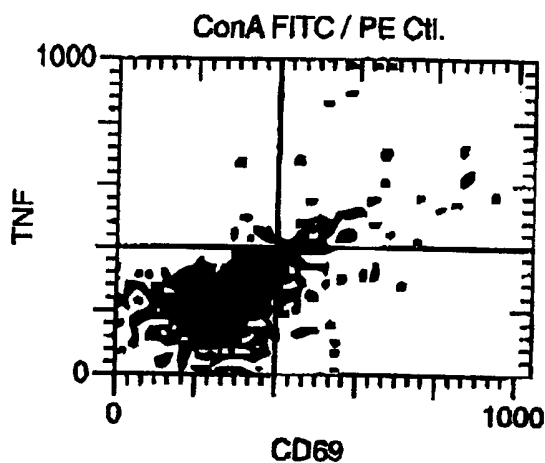
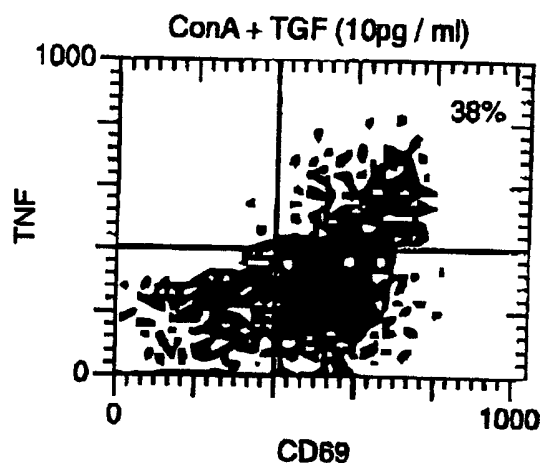
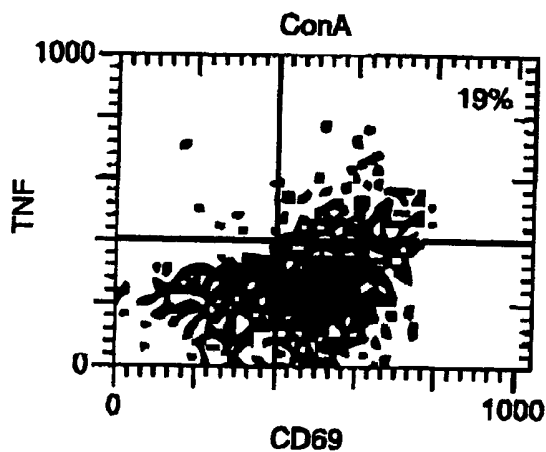
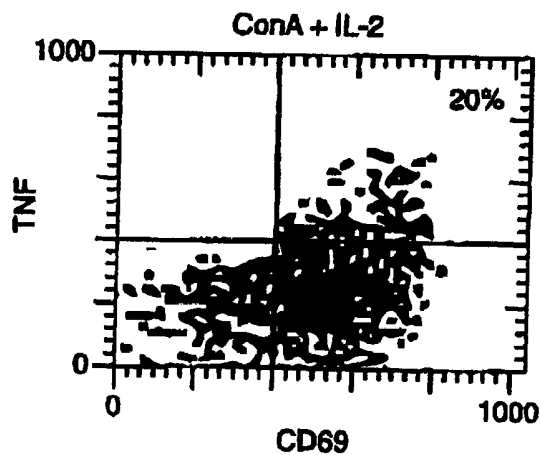
7. A kit according to claim 6 further comprising written instructions for the method of treating.

8. A kit according to claim 6 wherein said dose is contained within said cell treatment container.
9. A kit according to claim 6 wherein said dose is in a lyophilized form.
10. A kit according to claim 6 wherein said cell treatment container further comprises at least one reagent.
- 5 11. A kit according to claim 6 wherein said cell treatment container further comprises a sampling port to enable the removal of a fraction of said cells for analysis.
12. A kit according to claim 6 further comprising an exit port adapted to enable transport at least a portion of said cells to a recipient patient.
13. A kit according to claim 6 wherein said suppressive composition is a mixture of IL-10 and TGF- β .

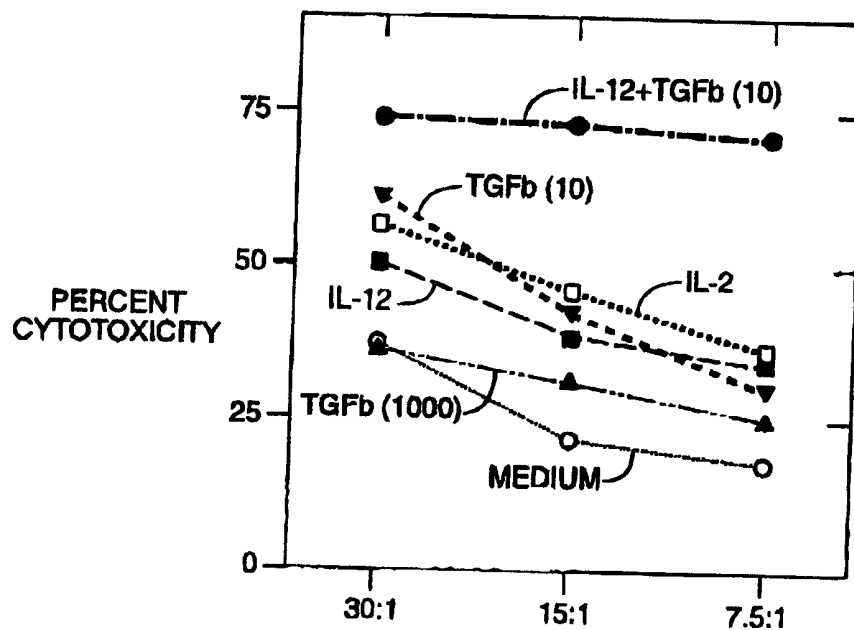
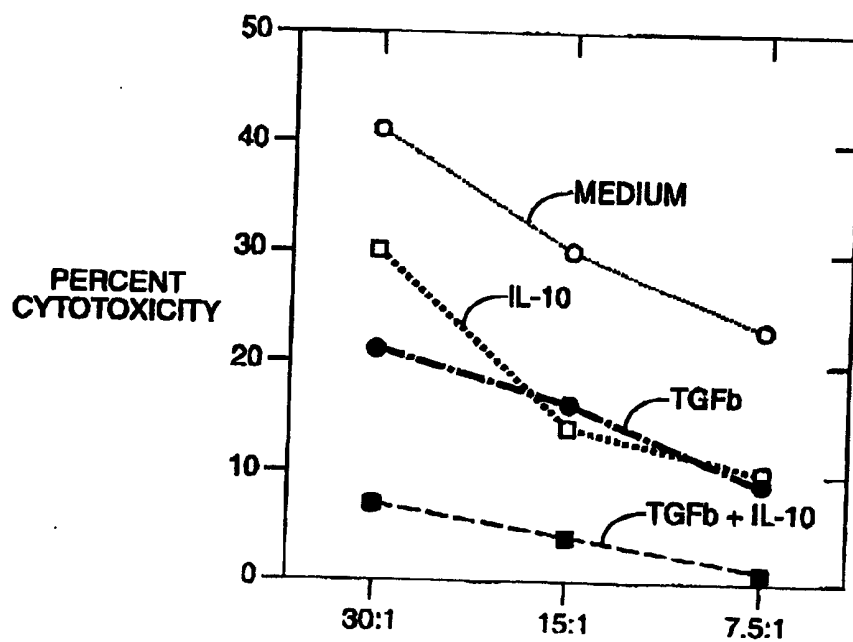
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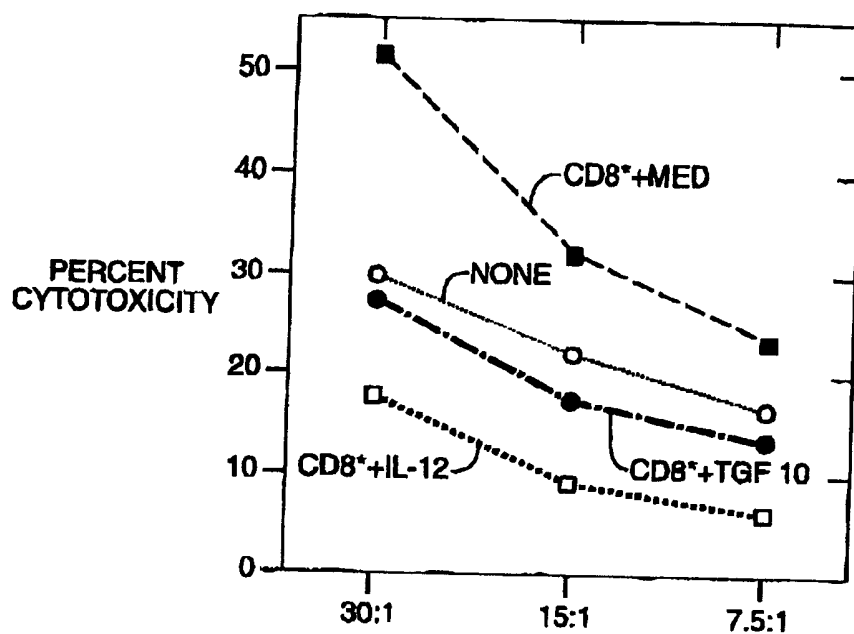
2 / 4

**FIG. 2A****FIG. 2B****FIG. 2C****FIG. 2D**

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**FIG. 4A****FIG. 4B**

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**FIG. 4C**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/04630

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/00 A61K38/18 A61K38/19 A61K38/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 42324 A (SCHERING CORP) 13 November 1997 (1997-11-13)	1,6-8, 10-12
Y	page 1, line 1 - page 4, line 26 page 12, line 1 - page 15, line 20; example 8 page 52, line 35 - page 53, line 27 page 34, line 18 - page 36, line 7 ---	2-5,9,13
X	PAWELEC G ET AL: "Cytokine modulation of TH1/TH2 phenotype differentiation in directly alloresponsive CD4+ human T cells." TRANSPLANTATION, (1996 OCT 27) 62 (8) 1095-101. , XP002113832	1,6-8, 10-12
Y	Abstract; Materials and methods starting at page 1096; Figures 1-4 --- -/-	2-5,9,13



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

31 August 1999

Date of mailing of the international search report

10.09.99

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/04630

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HALVERSON D.C. ET AL: "In vitro generation of allospecific human CD8+ T cells of Tc1 and Tc2 phenotype." BLOOD, (1997) 90/5 (2089-2096). , XP002113833	1,6-8, 10-12
Y	Summary and "materials and methods" starting at page 2089	2-5,9,13
Y	--- OSWALD I P ET AL: "IL-10 SYNERGIZES WITH IL-4 AND TRANSFORMING GROWTH FACTOR-BETA TO INHIBIT MACROPHAGE CYTOTOXIC ACTIVITY" JOURNAL OF IMMUNOLOGY, vol. 148, no. 11, 1 January 1992 (1992-01-01), pages 3578-3582, XP002062210 ISSN: 0022-1767 abstract	4,13
Y	--- WO 93 17698 A (SCHERING CORP) 16 September 1993 (1993-09-16) page 1, line 8-26 page 3, line 14 - page 4, line 5	4,13
T	--- ZELLER, J. C. (1) ET AL: "Ex vivo IL10 and TGF - beta act synergistically to induce CD4+ alloantigen-specific tolerance resulting in diminished graft-versus-host disease in vivo." FASEB JOURNAL, (MARCH 12, 1999) VOL. 13, NO. 4 PART 1, PP. A614. MEETING INFO.: ANNUAL MEETING OF THE PROFESSIONAL RESEARCH SCIENTISTS FOR EXPERIMENTAL BIOLOGY 99 WASHINGTON, D.C., USA APRIL 17-21, 1999 , XP002113834 abstract -----	1-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/04630

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 2-5
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 2-5 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 2-5

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/04630

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